

ADENOSINE RECEPTOR AGONIST PD 81,723 PROTECTS AGAINST SEIZURE/*STATUS EPILEPTICUS* AND NEUROPATHOLOGY FOLLOWING ORGANOPHOSPHATE EXPOSURE

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ABSTRACT

A novel approach to broad-specificity medical countermeasure against chemical warfare nerve agent (CWNA) poisoning is by preventing excessive accumulation of ACh attributable to AChE inhibition of CWNA exposure. The effectiveness of various partial adenosine A₁ receptor agonists as post-CWNA exposure (1 hour) neuroprotectants was investigated in the study using a rat seizure/*status epilepticus* model that employs the organophosphate diisopropylfluorophosphate, a surrogate comparable to the nerve agent soman. Physiological data collected via radiotelemetry demonstrates that the adenosine receptor agonist PD 81,723 protects against *status epilepticus* and neuropathology resulting from DFP exposure and can potentially be used for treatment against CWNA exposure.

INTRODUCTION

Exposure to organophosphate (OP) chemical warfare nerve agents (CWNAs) perturbs cholinergic neurotransmitter function and, subsequently, glutamatergic function. The resulting hypercholinergy leads to neurodegenerative conditions manifested by uncontrolled seizures/*SE*, which can be life threatening if not properly treated. However, the sequence of events following OP intoxication, which induces seizure/*SE* and permanent neuropathological damage, abnormal physiological function, neurobehavioral deficits, and potentially death, is not clearly known. Seizure/*SE* is sustained by excitatory amino acid release which activates NMDA receptors and voltage activated calcium channels. Calcium and other ionic changes lead to the generation of reactive oxygen species via activation of nitric oxide synthase, uncoupling of mitochondrial phosphorylation, and activation of lipases, proteases, endonucleases, and catabolic enzymes that eventually lead to cell death.

Current emergency treatment of acute OP poisoning, which consists of combined administration of an AChE reactivator (an oxime), a muscarinic ACh receptor antagonist (e.g. atropine), and an anticonvulsant (e.g. diazepam), is not general and does not prevent neuronal brain damage and its resulting incapacitation [1-7]. Diazepam terminates seizure/*SE* when it is administered soon after the CWNA exposure, but it is less effective at later time points, possibly due to the rapid modulation of GABA receptors during *SE* that result in pharmacoresistance [8]. The seizure/*SE*-induced brain damage is also refractory to pretreatment of muscarinic antagonist, atropine. Therefore, generic and more effective drugs that can protect against various CWNAs are essential for medical countermeasure when the nature of threat is not known.

A new, generic therapeutic approach for CWNA exposure is the pharmacological reduction of the release of ACh, the neurotransmitter which accumulates in brain and muscles following CWNA exposure. Preceding evaluations of drugs that potentially inhibit ACh release revealed that, based on the criteria a) rapid penetration in the brain, b) fast action, and c) effectiveness in both central and peripheral cholinergic system, adenosine A₁ receptor agonists are very suitable candidates. Adenosine, under physiological conditions, diminishes metabolic demand and increases nutrient supplies for metabolically active or stressed cells, thus playing an important role in the maintenance of local homeostasis. Adenosine also has

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a specific neuroprotective function under circumstances of enhanced neuronal activity, which is thought to be a result of postsynaptic modulatory effects on K^+ and Cl^- channel activity via the postsynaptic A1 receptor. This limits evoked depolarization and results in decreased activation of voltage-dependent Ca^{2+} channels and NMDA (N-Methyl D Aspartate) receptor ion channels, through which Ca^{2+} enters cell bodies [9]. Adenosine also inhibits the effects of adenylate cyclase and phospholipase C, which might contribute to its neuroprotective properties. Recently, the adenosine-induced mechanism has been attributed to the acquisition of neuronal ischemic tolerance. Furthermore, activation of the presynaptic A1 receptor by selective agonists results in a reduction of neurotransmitter ACh, aspartate, and glutamate release in several systems, consequently reducing convulsive activity [10-13].

These A1 receptor agonists are also effective in attenuating SE and kainate-induced convulsions, both in vitro and in vivo. Therefore, early administration of A1 receptor agonists should prevent or moderate ACh-induced onset of seizure activity and excitatory amino acid (EAA) recruitment. Moreover, the positive-feedback loop that reportedly exists between glutamate and ACh release, which amplifies the release of both neurotransmitters, is intervened in this way [16]. Administration of A1 receptor agonists during a later phase should be still effective because they inhibit EAA-induced neuronal damage by decreasing extracellular levels of EAA and by modulating the NMDA receptor via K^+ and Cl^- channels. Thus, A1 receptor agonists could be very effective in the early treatment of OP poisoning.

It has been shown that A1 receptor agonists N6-cyclopentyl adenosine (CPA) and 5'-N-ethylcarboxamido-adenosine (NECA), administered by intramuscular injection 1 min following subcutaneous soman poisoning (1.5-2 LD50) in rats, has resulted in prevention or postponement of chewing, salivation, convulsive activity and respiratory distress (symptoms produced by excessive ACh) [14]. This was associated with low levels of extracellular ACh in the brain and dramatic decrease in the mortality rate [15]. Although lower doses of CPA in combination with physostigmine, hyoscine, and HI-6 has been shown to produce significant protection against soman lethality and incapacitation, CPA produces adverse cardio-depressant effects with the stimulation of the A1 receptors in the heart. For that reason, various analogues of CPA that are partial agonists and exhibit greater organ selectivity without cardiovascular side-effects need to be tested for protection against CWNA exposure.

The goal of the present study is to evaluate adenosine receptor agonist PD81, 723 [(2-amino-4, 5-dimethyl-trienyl) [3-(trifluoromethyl)phenyl]methanone] for post-exposure protection against seizure/SE and neuropathology. PD 81, 723 has been shown to allosterically enhance agonist binding and function of the adenosine A1 receptor while simultaneously inhibiting the binding of antagonists/inverse agonists. The present study utilized radiotelemetry and a recently developed rat DFP model of seizure/SE model for evaluation. The data collected reveals that post-exposure treatment with PD 81,723 significantly reduced seizure/SE and the subsequent neuropathology, suggesting that it is a potent neuroprotectant against seizure/SE following organophosphate exposure.

MATERIALS AND METHODS

Animals: All animal experiments were performed in accordance with principles stated in the *Guide for Care and Use of Laboratory Animals and Animal Welfare Act*. Adult male Sprague-Dawley rats (225-250 g, Charles River Laboratories, MA) were housed under standard conditions with 12 h light/dark cycle and food and water available *ad libitum*. The rats were quarantined after arrival for 1 week prior to surgical implantation of the radiotelemetry probes.

Surgical implantation of the radiotelemetry probes: The radiotelemetry probe FL-50 EET and the radiotelemetry system were purchased from Data Sciences International (St. Paul, MN). The probes were sterilized and handled according to the instructions of the manufacturer. One week after the arrival of the animals, each was anesthetized by isoflurane (2% isoflurane and 1.5% oxygen) and placed on a stereotax for implantation of the probes. After administration of Bupivacaine analgesic, a small incision was made along the midline of the dorsal side of the head and also along the midline of the dorsal side of the body 3 inches cranial to the base of the tail. The two cortical electrodes and control electrode of the radiotelemetry probe were tunneled subcutaneously from the dorsal body incision to the upper surface of the skull in the presence of local anesthetic Lidocaine. The two cortical electrodes were implanted on 1 mm circumference holes, each made 3 mm on either side of the center and 3 mm above the lambdoid

suture. The reference electrode was placed on the right side, 3 mm from the sagittal suture and 3 mm above the coronal suture. The electrodes were immobilized using screws and dental acrylic. The two electrodes for ECG recordings were tunneled and placed to the right pectoral muscle and to the lower side of the xiphoid process, respectively. The body of the telemetry probe was placed on the dorsal left quadrant of the animals and sutured into place. Each rat was administered with Bupivacaine post-surgically at 6 to 12 h intervals for 24 h.

DFP-induced seizure/SE: On the day of the experiment, the rats housed in individual cages were placed on radiotelemetry receivers, and the baseline EEG, ECG, temperature, and physical activity were recorded for 30 min. The rats were administered pyridostigmine bromide (0.26 mg/kg, i.m.). Thirty minutes later, freshly diluted DFP in ice-cold saline (4 mg/kg, s.c.) was administered, followed by a mixture of atropine and 2-PAM (0.2 mg/kg and 25 mg/kg, i.m., respectively) at 31 min. DFP was always kept on ice, and the dilution was done using a Hamilton syringe (Australia). Also the drugs 2-PAM, atropine, and PB needed to be prepared freshly once in 2-3 months since each drug's activity has potential to go down during storage. The animals were then placed back on the receiver after each injection, and the data was continuously monitored and recorded for 24 h. Most of the animals showed proneness to undergo seizure/SE under these conditions; however, in atypical cases where an animal did not produce seizure/SE in 10 min, that animal was immediately administered an additional 0.5 mg/kg of DFP to induce seizure/SE.

Post exposure treatment with PD 81, 723: PD 81, 723 was obtained from (Tocris Cookson Inc., Ellisville, MO). The drug was prepared freshly at a concentration of 10 mg/mL in DMSO and sterilized by filtration. Sixty minutes after the onset of seizure/SE, the drug was administered subcutaneously in a volume of approximately 50 micro liters. EEG, ECG, temperature, and physical activity were continuously monitored for 24 h.

Survivability: The overall health and survivability of the rats within the 24 h duration of the experiments were also recorded to evaluate the protective effect of PD 81, 723.

Data analysis: The experimental data was analyzed using Dataquest ART 2.3 (DSI International, MN) and plotted over time. EEG data was analyzed using waveform analysis, and the latency of seizure termination was determined based on the time required to reduce the amplitude of seizure spikes ≤ 2 standard deviation of basal EEG amplitude recorded at the beginning of each experiment. Latency period also corresponded to the time period where there was no further seizure development ≥ 10 sec. A power spectrum of EEG data was also performed using Dataquest ART2.3 at representative time points. Heart rate was calculated directly from the ECG recordings using Dataquest ART 2.3. Physical activity is shown as spikes in Dataquest ART. The body temperature was directly plotted over time in Celsius.

Neuropathology: Animals surviving 24 h were deeply anesthetized with 75 mg/kg pentobarbital and euthanized by exsanguinations. The brain was removed and fixed with 4% formaldehyde. Serial 2 mm sections were cut using a slicing chamber, and the anteroposterior level of the sections were selected according to the stereotaxic coordinates of the rat brain atlas of Paxinos and Watson (-3.0 and -4.0 from Lambda reference). Blocks were sectioned at 5 μ m and stained by hematoxylin and eosin (H&E). The grades used are based on histological analysis of H&E stained brain sections, using the criteria of McDonough, et al, [16;17] [18] with slight modifications as follows: 0 – no neuronal necrosis, 1 – necrosis of 1-10% of neurons, 2 – necrosis of 11-25% of neurons, 3 – necrosis of 26-50% of neurons, and 4 – necrosis of $> 50\%$ of neurons

Neuronal damage was quantitated for each brain and for each staining method using 26 high magnification fields representing 4 regions of the hippocampus to examine neuronal damage. Thirteen fields were from each half of the brain, representing 3 fields of CA1, 3 fields of CA3, 5 fields of the dentate gyrus, and 2 fields of the polymorphic layer (hilus). Each field was photographed, and the number of degenerate or dead neurons for each field was determined through visual examination of photomicrographs by a board-certified veterinary pathologist in a blind fashion. Based on previous studies, damaged neurons demonstrated by H&E are expected to exhibit hypereosinophilic cytoplasm and hyperchromatic nuclei or, less frequently, to be shrunken and have pyknotic nuclei. All raw data was entered into an Excel spread sheet. The mean number of damaged neurons for each of the 4 regions of

hippocampus will be determined and other statistical analyses will be done to compare experimental groups.

Functional observation battery tests: Functional observation battery tests consist of 25 measures of sensorimotor and autonomic functions [19-21]. Firstly, the animals were observed while undisturbed in the cage. Animal posture, palpebral closure, and presence or absence of convulsions was scored. If convulsions were present, they were further categorized. Following observation in the cage, each animal was removed and briefly held in the hand. Ease of removal and handling, skin and fur abnormalities, lacrimation, and nose secretion were recorded. Reflex testing consisted of the rat's response to the frontal approach of a blunt object, a touch of an object to the posterior flank, and an auditory click stimulus. Reactivity of the animal to a pinch on the tail, the ability of its pupil to contract, and the dexterity of the animal as it rights itself were also assessed.

Blood and Brain AChE assay: At the end of the study, the animals were deeply anesthetized with 75 mg/kg pentobarbital before blood was collected by cardiac puncture. The blood was then immediately frozen for future whole blood cholinesterase assays. Blood AChE was measured (WRAIR-microassay) spectrophotometrically at 410 nm by the method of Ellman et al [22] using acetylthiocholine iodide as the substrate. Since there is an excellent correlation between brain and spinal cord AChE activity [23], the spinal cord was obtained and stored for further analysis. The cervical segment was homogenized in the presence of Triton X-100 or T-PER (Sigma Chemical Co.) in ice and centrifuged. Then the total AChE activity was measured by WRAIR assay. Finally, protein concentration was determined by Lowry's method or by BCA protein assay reagent (Pierce Chemical Co, Rockford, IL).

RESULTS

Post exposure treatment with PD 81,723 increases the survivability: The rat model of seizure/SE involves pyridostigmine bromide (0.026mg/kg i.p.), followed 30 and 31 min later, respectively, by DFP (3.8-4.0 mg/kg s.c.) freshly diluted in ice-cold saline and a mixture of atropine (0.2 mg/kg i.m.) and 2-PAM (25 mg/kg i.m.). Seizure/SE induced by DFP occurs approximately 8-15 min after administration and is accompanied, if not heralded, by predictable muscarinic symptoms and behavioral signs (e.g. tremors, salivation). Although most of the animals experience seizure/SE following DFP exposure, few animals die shortly after the onset of seizure/SE. with the estimated survivability of the animals 24 h after DFP exposure being approximately 61% (Table 1). Administration of PD 81, 723, 1 mg/kg s.c., enhances the survivability of DFP treated animals to 100%. Increasing the concentration of PD 81,723 to 1.5 mg/kg also decreased the mortality rate of DFP treated animals. However, pathology reports indicate that neuroprotection at PD 81, 723, 1.5 mg/kg, s.c., was not as effective as PD 81,723, 1.0 mg/kg, s.c. (Table 2). Further increase of the dose of PD 81, 723 to 2.0 mg/kg, s.c., did not increase the survivability of DFP treated animals (Table 1) and also did not protect effectively against neuropathology (Table 2), indicating that higher dose of PD 81, 723 is toxic to the animals.

TABLE 1. Animal survivability following post exposure treatment with PD 81, 723

Drug	Dose(mg/kg)	Survivability (%)
Saline		100
DFP	3.8-4.0	61
PD 81,723	1.0 (7)	100
	1.5 (3)	100
	2.0 (4)	50

Table 2. Neuronal damage reduction in different regions of the brain following treatment with PD 81,723

Treatment	Neocortex	Hippocampus	Thalamus	Pyr. Cortex	Amygdala	Caudate
Saline	0.00	0.00	0.00	0.00	0.00	0.00
DFP	1.93	1.64	2.43	2.00	2.43	0.36
PD 1.0mg/kg	1.66	0.75	2.00	0.75	2.17	0.00
PD 1.5mg/kg	1.50	2.50	2.00	1.50	2.00	0.00
PD 2.0mg/kg	2.00	2.33	3.00	1.67	2.33	0.67

No damage was found in cerebellum and brainstem of DFP treated rats. Grades are based on histologic analysis of H&E-stained brain sections, using the criteria of McDonough, et al., with slight modifications, as follows: 0 – no neuronal necrosis, 1 – necrosis of 1-10% of neurons, 2 – necrosis of 11-25% of neurons, 3 – necrosis of 26-50% of neurons, 4 – necrosis of > 50% of neurons

Post exposure treatment with PD 81,723 protects against seizure/SE: Next, the protective effect of PD 81,723 was evaluated by analyzing the EEG recordings. Initially, the EEGs were compared in an hourly format to demonstrate an overall picture of the EEG recordings. The y-axis was kept constant (-1.0 to +1.0 mV) in all the animals for comparison. Twenty-four h of recording is shown in Figure 1, with each line representing an hour of recording. The timings of all the recordings are similar. Firstly, the 30 min baseline recording can be seen, followed by DFP and the atropine + 2-PAM combination at 60 and 61 min, respectively. Then, 60 min after the onset of seizure/SE, the animals were administered with the adenosine receptor agonist PD 81,723. As can be seen in Figure 1 A, saline administration does not induce any high voltage spikes in control rats. Conversely, treatment with DFP, 4.0 mg/kg s.c., (Figure 1 B) produces continuous, high voltage spikes that indicate SE. Although the spikes gradually decrease in amplitude after 6 – 8 h following exposure, SE is never completely eradicated in DFP treated animals.

Due to the repetitive discharges in DFP treated animals, it was difficult to determine the latency of seizure termination. However, in rats treated with PD 81,723 (Figure 1 C), there was a significant reduction in the amplitude of the seizure when compared to that of the animals that were only exposed to DFP and not treated with PD 81,723, henceforth referred to as DFP controls (Figure 1 B). The number of spikes per time was also significantly reduced in the PD 81,723 treated animal, indicating that PD 81,723 shows anti-epileptic activity. The time required for 50% reduction of the seizure amplitude in PD 81,723 treated animals was much shorter than that of the DFP treated controls.

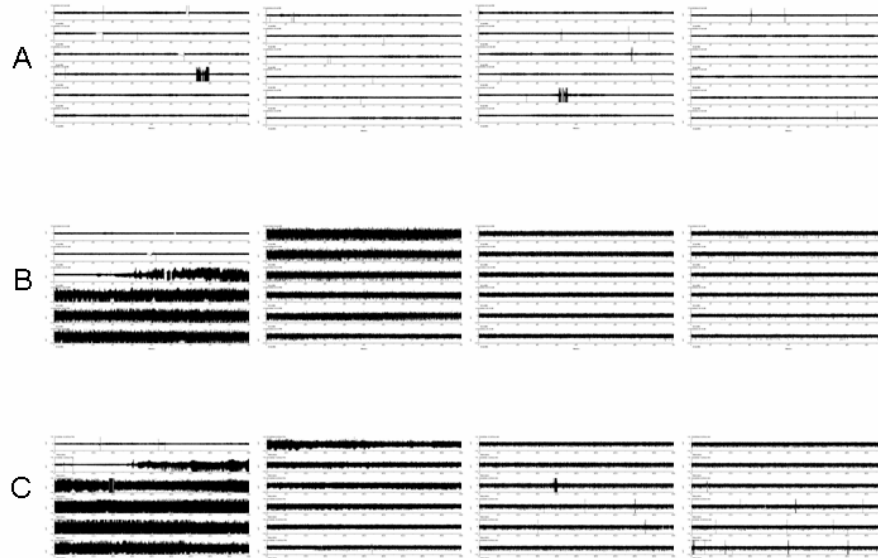


FIGURE 1. PD 81,723 protects against seizure/SE in rat DFP model of seizure/SE. A. Baseline EEG of control rats implanted with telemetry probes is recorded for 30 min. The rats are at that time administered pyridostigmine bromide (0.026 mg/kg, i.m.). After an additional 30 min, saline is administered, followed by a mixture of atropine sulfate and 2-PAM (0.2 mg/kg and 25 mg/kg, respectively, i.m.) 1 min later. EEG is continuously recorded for 24 h. The EEG is recorded continuously for 24 h with the y-axis range as -1 to +1 mV and each line representing 1 h of EEG recordings. B. Rats are administered pyridostigmine bromide (0.026 mg/kg, i.m.) 30 minutes from the start of recording. DFP (4 mg/kg, s.c.) freshly diluted in 300 μ l phosphate buffered saline is administered 30 minutes following the pyridostigmine. One minute later the mixture of atropine sulfate and 2-PAM (0.2 mg/kg and 25 mg/kg, respectively, i.m.) is injected. C. A representative EEG recording of rats exposed to DFP as described above; however, the animal is treated with PD 81,723 60 min after the onset of seizure/SE. Note that the seizure amplitude and frequency is significantly decreased after PD 81,723 administration.

PD 81,723 reduces neuropathology following DFP exposure: Histopathology of the brain of PD 81,723- and DFP-treated animals that survived 24 h was analyzed in a blind fashion. DFP was capable of inducing neuropathology in the rat seizure/SE model (Table 2). The lesion, involving neuronal necrosis that consisted of shrunken pyknotic/necrotic individual neurons, was scored according to McDonough et al [24;25] and graded on a scale of 0-4 (Figure 2). Table 2 displays the average brain lesion scores for individual brain regions in PD 81,723-treated animals compared to animals exposed to DFP without treatment. Saline control animals did not show any neuropathology. DFP treated animals surviving 24 h showed severe and frequent damage at the amygdala, thalamus, and cortex. Interestingly, pathology reports showed that the DFP-treated animals that died shortly after the onset of seizure did not show any neuropathology, indicating that longer periods of seizure/SE are indeed required to initiate neuropathology. Animals treated with 1 mg/kg PD 81, 723 showed significantly lower neurodegeneration when compared to animals exposed to DFP but not treated 1 h post onset of seizure. The hippocampus, pyriform cortex, and caudate nucleus were protected more effectively than the amygdala, thalamus, and neocortex.

For animals treated with 1.5 mg/kg PD 81,723, the neuropathology was an improvement compared to DFP control animals, but its neuroprotective effects in the hippocampus and pyriform cortex were not as strong as animals treated with 1.0 mg/kg PD 81,723 (Table 2). Neuropathology reports for animals treated with 2.0mg/kg PD 81,723 were similar to DFP control animals. Therefore, it is suggested that the protective effect against neuropathology was reduced by further increase in the dose of PD 81, 723.

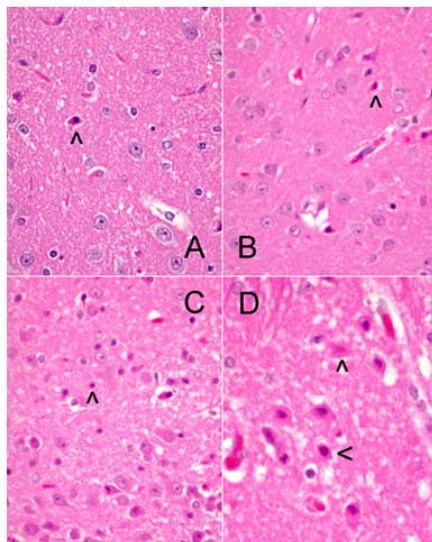


FIGURE 2. Neuropathology demonstrating scores of brain lesion in the DFP model of seizure/SE. A representation of neuronal damage scores to indicate the level of lesion in DFP treated animals that produce seizure/SE and survive more than 24 h is shown. A. 1-10% neuronal damage; B. 11-25% damage; C. 26-50% damage; and D. greater than 50% neuronal damage. Brain regions with most severe brain damage are indicated by dark staining condensed chromatin. Note the argyrophilic, darkly stained damaged neurons in DFP treated rat brain samples. In the DFP model, more damage is observed at CA1 of the hippocampal region compared to CA3. There was no neuronal damage in control rats exposed to saline.

Behavior of animals treated with PD 81,723: DFP exposure results in predominantly cholinergic symptoms with a variety of physical signs of intoxication and delayed neurotoxic effects [26;27]. In the present studies, animals treated with PD 81,723 were subjected to a series of functional observation battery tests [28]. A summary of these results are shown in Table 3. Following treatment with PD 81,723, many of the muscarinic symptoms are normalized. Head tremors and clonic and tonic jerks are also reduced. The coordination signs are more intact with animals treated with PD 81,723 when compared to animals not treated after 1 h of seizure. Thus, animals treated with PD 81,723 following exposure to DFP have reduced incidence of neurobehavioral abnormalities.

TABLE 3. Behavioral measurements of animals treated with PD 81, 273 compared to DFP controls.

Behavioral sign	DFP	PD
Observation		
Posture	Standing with head bobbing	Standing
Catch	Passive	Normal
Handling	Passive	Normal
Muscular tonus	Hypertonia	Normal
Lacrimation	Some	Slight
Endo-/exophthalmus	Exo-	Normal
Skin	Cold	Normal
Salivation	Some	None
Nose secretion	Some	None
Clonic/Tonic Movements	Some repetitive movements and jerks	Normal
Gait	Impaired—hunched body, walks on tiptoes	Walks on tiptoes
Mobility	Impaired	Normal
Arousal/unprovoked activity	Low	Reduced
Approach Response	None	Slow
Touch Response	Slow to none	Normal
Click Response	None	Normal
Tail-pinch	None	Normal
Righting Reflex	Uncoordinated	Slightly uncoordinated

Heart rate in PD 81, 723 treated animals: Following DFP treatment heart rate is slightly increased from the baseline level (approximately 400 beats per min) but still within the normal limits of 300-500 beats per minute (Figure 3 B and C). However, with time the heart rate of DFP treated animals gradually decreases lower than baseline, reaching the lower extent of normal range, approximately 340 beats/min, after 7 h (Figure 3 B). The most drastic decrease in heart rate for animals treated with DFP, which occurs at approximately 6.5 hours after the start of recording, is not as severe in the PD 81,723 treated animals (Figure 3 C) as it is in the DFP control animals (Figure 3 B). Also there is less variation in heart rate for the PD 81,723 treated animals throughout the 24 h period in comparison to the DFP control animals (Figure 3 C and B, respectively).

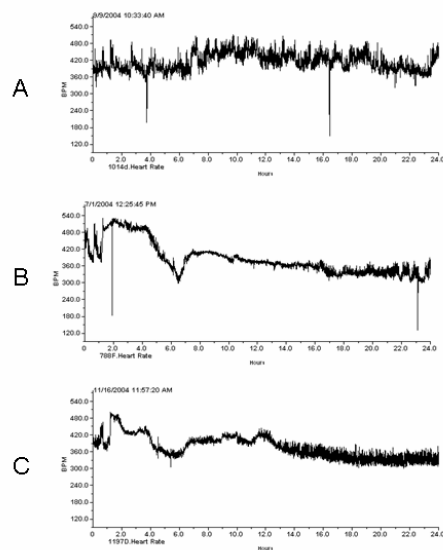


FIGURE 3. Heart rate in animals treated with PD 81,723 following exposure to DFP. A. Rats implanted with telemetry probes were administered pyridostigmine bromide (0.026 mg/kg, im) followed by 300 μ l saline and a mixture of atropine and 2-PAM (2 mg/kg and 25 mg/kg, im) was injected. The heart rate was recorded continuously. B. Rats were administered pyridostigmine bromide (0.026 mg/kg, i.m.) followed by DFP (4 mg/kg, s.c.) freshly diluted in 300 μ l phosphate buffered saline. One minute later a mixture of atropine sulfate and 2-PAM (2 mg/kg and 25 mg/kg, respectively, i.m.) was injected. The heart rate was recorded continuously. C. Rats were administered pyridostigmine bromide (0.026 mg/kg, i.m.) followed by DFP (4 mg/kg, s.c.) freshly diluted in 300 μ l phosphate buffered saline. One minute later a mixture of atropine sulfate and 2-PAM (2 mg/kg and 25 mg/kg, respectively, i.m.) was injected, and the heart rate was recorded continuously. Sixty min after onset of seizure, the animals were treated with 1 mg/kg PD 81,723, s.c. The y-axis is beats/min, and the x-axis represents 24 h of recordings. Note that the heart rate of PD 81,723 treated animals is more unvarying than that of the DFP controls

Physical activity is improved following treatment with PD 81, 723: Physical activity in the absence of seizure is an indication of the level of recovery in the animals. Movement of each animal is recorded as spikes. The animal movement is higher during night time (from 7 h to 19 h after the treatment). The physical activity is significantly decreased in the DFP control animals when compared to the saline control animals (Fig 4 B and A, respectively). The “quiet period” observed 1 -12 h after DFP administration is reduced almost completely in PD 81,723 treated animals (Figure 4 C). PD 81,723 treated animals also show more occurrences of spikes than the DFP control animals, indicating that treatment with PD 81,723 allows for the animal to improve its physical activity post DFP exposure.

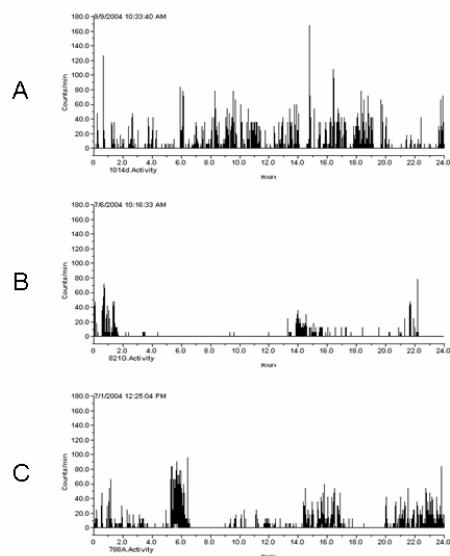


FIGURE 4. Physical activity is improved in PD 81,723 treated animals following DFP exposure. A. Rats implanted with telemetry probes were administered pyridostigmine bromide (0.026 mg/kg, i.m.), followed by 300 μ l saline and a mixture of atropine sulfate and 2-PAM (2 mg/kg and 25 mg/kg, i.m.). The physical activity was recorded continuously. B. Rats were administered pyridostigmine bromide (0.026 mg/kg, i.m.), followed by DFP (4 mg/kg, s.c.) freshly diluted in 300 μ l phosphate buffered saline. One minute later a mixture of atropine sulfate and 2-PAM (2 mg/kg and 25 mg/kg, i.m.) was injected, and the physical activity was recorded continuously. C. Rats were administered pyridostigmine bromide (0.026 mg/kg, i.m.), followed by DFP (14 mg/kg, sc) freshly diluted in 300 μ l phosphate buffered saline. The atropine sulfate/2-PAM mixture (2 mg/kg and 25 mg/kg, respectively, i.m.) was injected 1 min later, and the physical activity was recorded continuously. The animals were treated with 1 mg/kg PD 81,723 60 min after the onset of seizure/SE. The y-axis is measured in counts/min, while the x-axis represents 24 h of recording. Note that the activity of DFP treated animals was significantly reduced, but the PD 81,723 treatment improved the physical activity.

Body temperature in PD 81, 723 treated animals: Administration of DFP (4.0 mg/kg, s.c.) strongly reduces the body temperature from $36.8 \pm 1^\circ\text{C}$ to $30 \pm 2^\circ\text{C}$ (Figure 5 B). A small dip can be seen in the temperature 30 min after the administration of DFP that returns back to normal within 2 h. A major decrease in the temperature is observed 6 h after the start, and peak reduction of body temperature is observed after 12-16 h of DFP exposure. Very gradually, the temperature increases in the direction of normal towards the end of the 24 h observation period. In PD 81,723 treated animals (Figure 5 C), a drop in the temperature similar to that in the DFP controls was observed after 30 min. However, unlike the DFP controls, this initial temperature drop is not returned to normal in PD 81,723 animals. The peak reduction in the temperature is observed at 6 h and is lower ($32.5 \pm 2^\circ\text{C}$) in PD 81, 723 treated animals compared to DFP control. The temperature gradually tends to move towards normal in PD 81,723 treated animals. Overall in PD 81,723 treated animals (Figure 5 C), the body temperature is reduced to a lesser extent when compared to DFP controls (Figure 5 B). Moreover, the rate of normalization of the body temperature was better in PD 81,723 treated animals.

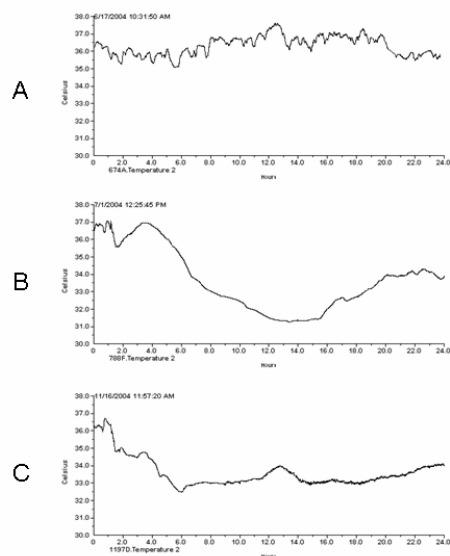


FIGURE 5. Body temperature in animals treated with PD 81,723 following exposure to DFP. A. Rats implanted with telemetry probes were administered pyridostigmine bromide (0.026 mg/kg, i.m.) followed by 300 μ l saline and a mixture of atropine sulfate and 2-PAM (2 mg/kg and 25 mg/kg, respectively, i.m.). The body temperature was recorded continuously for 24 h. B. Rats were administered pyridostigmine bromide (0.026 mg/kg, i.m.). After 30 min, DFP (4 mg/kg, s.c.) freshly diluted in 300 μ l phosphate buffered saline was administered, followed by the mixture of atropine sulfate and 2-PAM (2 mg/kg and 25 mg/kg, i.m.) 1 min later. The temperature was recorded continuously. C. Rats were administered pyridostigmine bromide (0.026 mg/kg, i.m.), followed by DFP (4 mg/kg, s.c.) freshly diluted in 300 μ l phosphate buffered saline after 30 min and the atropine and 2-PAM mixture (2 mg/kg and 25 mg/kg, i.m.) after 31 min. The temperature was recorded continuously. Animals were then treated with 1 mg/kg PD 81,723 60 min after the onset of seizure/SE. The y-axis is measured in degrees Celsius, and the x-axis represents 24 h of recording. Note that, although the temperature of PD 81,723 treated animal drops, especially at the critical 6 h time point mentioned in the text, it begins to move towards normal as time progresses.

Effect of combined therapy with PD 81,723 and diazepam following exposure to DFP: To deliver optimum neuroprotection in animals exposed to DFP net, we investigated the effect of combination therapy with PD 81, 723 and diazepam. Preliminary results indicated that combination of PD 81, 723 (1mg/kg) and diazepam (10 mg/kg) greatly reduced seizure/SE in the rat; however, the sedative, respiratory depression, and ataxia effects of diazepam, in combination with PD 81, 723, also greatly decrease heart rate, temperature, and physical activity to levels harmful to the rat. More studies with lower doses of diazepam or non-sedative and non-respiratory suppressant partial inhibitors of benzodiazepines are required to evaluate the efficacy of combination therapy.

DISCUSSION

Seizure/SE is a major, life threatening symptom of chemical warfare nerve agent exposure. If treatment is not initiated early, the seizure/SE may lead to irreversible neuronal brain damage and neurobehavioral deficits. When the nature of threat is not known, a drug that works well against various CWNAs is required. In this study we investigated PD 81,723, an adenosine receptor A1 agonist that blocks the central release of ACh, for protection against seizure/SE following exposure to the organophosphate DFP, a surrogate of soman and sarin. Utilizing radiotelemetry and a DFP-induced rat seizure/SE model previously developed in our laboratory (Crawford et al., manuscript submitted), we show that PD 81, 723 protects against seizure/SE and neuropathology, as well neurobehavioral deficits, following exposure to DFP.

It has been reported that SE is caused by loss of the adenosine anticonvulsant mechanism [29]. In this sense supplementation of adenosine or activation of adenosine receptors should be significant in reducing seizure/SE, which is essential to protect against neuropathology. Prior research indicates that adenosine A1 receptor agonists could protect against seizure/SE following exposure to CWNAs. It has been shown that A1 agonists decrease the amount of agents delivered to target sites by inducing hypotension and bradycardia [15]. A1 receptor agonists inhibit adenylate cyclase and phospholipase, which promote neurodegeneration by generating second messengers. The adenosine-induced mechanism has been attributed for the acquisition of neuronal ischemic tolerance [30]. Adenosine A1 receptor agonists also suppress seizure in a pharmacoresistant model of epilepsy [31]. The A1 receptor attenuates traumatic rat hippocampal cell death [32] and is also implicated in suppressing peripheral inflammation [33].

With regard to CWNAs, it has been shown that A1 receptor agonists affect the inactivation of AChE in blood and brain by sarin [15]. Partial A1 receptor agonists inhibit sarin induced epileptiform activity in the hippocampal slice [34]. Pilot experiments with NECA and CPA treatment 1 min following soman (2LD₅₀) poisoning reduced extracellular ACh levels and postponed chewing salivation, convulsive activity, and respiratory distress, decreasing mortality rate dramatically [35;36]. Post exposure treatment (1 min) with CPA was highly effective against tabun and sarin [15] but was not against VX and parathion, mainly because it is the lower metabolism and increased stability of VX and parathion that lead to neurotoxicity. Yet, the present study is the first investigation evaluating the neuroprotective effect of adenosine receptor agonists by administering treatment 60 min after the onset of seizure/SE. We demonstrate that administration of adenosine receptor agonists at later time points decrease mortality and improve recovery following exposure to OP, as predicted by Van Helden. It should also be noted that all the previous experiments reported by Van Helden were done in the absence of PB, 2-PAM and atropine treatment. We, on the other hand, have investigated PD 81,723 in the presence of the above drugs. It is not known whether the PB, oxime, and atropine drug combinations may have synergistic effects or if the protective effect is due to additional effects of individual drugs. Further work is required to unravel the detailed mechanism of PD 81,723 mediated neuroprotection.

Interestingly, PD 81,723 also improves physical activity, prevents reduction of body temperature, and maintains stable heart rate. All these results are consistent with strong neuroprotection. One thing that stands out in the data collected in our research is the sharp change of heart rate and body temperature in DFP controls and, thus, adenosine receptor agonist treated animals 6 h after the start of the experiment. As a result many animals in this study treated with drugs other than PD 81,723 have died 6 h into the experiment. It is thought that, by preventing the drastic drop of temperature to lethal levels, PD 81,723 and other adenosine receptor agonists could exert greater neuroprotective properties. It is also thought that a second regimen of drugs to recover heart rate and temperature could improve the neuroprotection and survivability in organophosphate exposed animals.

In this study data shows that PD decreases the seizure/SE. Since significant epileptiform activity remains after the major reduction of DFP induced seizure/SE, it is difficult to establish seizure latency time period. Therefore, because seizure termination is important for neuroprotection, these results suggest that PD 81,723 should be administered in combination with an anticonvulsant to be more effective in neuroprotection. To this end we have begun to conduct experiments administering 10 mg/kg diazepam along with PD 81,723 60 min after the onset of seizure. Thus far it seems that this dose of diazepam in combination with PD 81,723 is toxic to animals. It seems that the toxicity is a consequence of severe temperature fall attributable to the anesthetic and other side effects of diazepam. Further reduction in the dose of diazepam to 3 mg/kg was not lethal but reduced the animal activity after 14 h. Nevertheless, these animals showed strong reduction in the amplitude and frequency of seizures compared to PD 81,723 treatment alone. These experiments need to be repeated with lower doses of diazepam or other anticonvulsants without sedative effects and respiratory depression.

Neuropathology data shows that treatment with PD 81,723 after 1 h of seizure/SE induced by DFP exposure exerts protective effect in all the different regions of the brain. Still, the extent of protection is different at different regions. The amygdala, thalamus, and pyramidal cortex were least protected, while the hippocampus, caudate nucleus, and neocortex were reported to be better protected with PD 81,723.

The lower protection at the thalamus and amygdala could be due to the high severity of damages in these regions following exposure to DFP. The DFP seizure model used in the evaluation of the efficacy is very reproducible and seems to be similar to chemical warfare nerve agent exposure in rats described by McDonough et al [8;18;25]. Neuropathology data shows that, similar to soman, the highest lesion was in the amygdala brain region following exposure to DFP. It appears that neuroprotection at amygdala and thalamus needs more potent drugs or new target strategies for treatment.

Blood AChE levels were significantly inhibited more than 65% in DFP treated animals compared to controls. This is consistent with the fact that more than 65% inhibition of AChE activity is required to induce seizure/SE. preliminary results show that the AChE activity in the PD 81,723 treated animals was slightly higher indicating that control of pathology is important for rapid recovery. Spinal cord AChE activity that is similar to brain AChE was also significantly inhibited by DFP and treatment with PD 81, 723 improves the enzyme activity.

CONCLUSION

In summary, this study suggests that the adenosine receptor agonist PD 81,723 is a neuroprotectant that can be administered after exposure to organophosphate to reduce neuronal death and minimize neurobehavioral deficits. An important aspect that requires more investigation is whether a combination of an anticonvulsant and PD 81,723 would produce an even greater improvement in neuroprotective and neurobehavioral effects. Furthermore, PD, 81,723 should be investigated with potent partial agonists of GABA that have no sedative property and thus do not cause respiratory depression. This combination may prove to be ideal for protection against exposure to CWNA when the nature of threat is not known.

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